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Towards feasible and scalable solvent-free enzymatic polycondensations: integrating robust biocatalysts with thin film reactions

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There is an enormous potential for synthesizing novel bio-based functionalized polyesters under environmentally benign conditions by exploiting the catalytic efficiency and selectivity of enzymes. Despite the wide number of studies addressing *in vitro* enzymatic polycondensation, insufficient progresses have been documented in the last two decades towards the preparative and industrial application of this methodology. The present study analyses bottlenecks hampering the practical applicability of enzymatic polycondensation and that have been most often neglected in the past, with a specific focus on solvent-free processes. Data here presented elucidate how classical approaches for enzyme immobilization combined with batch reactor configuration translate into insufficient mass transfer as well as limited recyclability of the biocatalyst. In order to overcome such bottlenecks, the present study proposes thin-film processes employing robust covalently immobilized lipases. The strategy was validated experimentally by carrying out solvent-free polycondensation of esters of adipic and itaconic acids. The results open new perspectives for enlarging the applicability of biocatalysts in other viscous and solvent-free synthesis.

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Introduction

In vitro enzymatic catalysed polymer synthesis has been extensively investigated in the last decades and lipases, in particular, demonstrated their efficiency in catalysing polycondensation and ring opening polymerization. The general concept of enzymatic catalysed condensation of polyols and diacids has been studied already in the '90^s and then transferred at industrial scale by Baxenden Chemicals (UK) for the production, later dismissed, of highly regular structures of polymers used in coating and adhesive applications.[1,2] Indeed, enzyme selectivity minimizes branching and enables the synthesis of functionalized polyesters characterized also by low polydispersity. [3-7]

An array of biobased and renewable monomers has been also employed for enzymatic polyester synthesis at laboratory scale. [8-11] The most important examples of bio-based polyols include: ethylene glycol, 1,2-propanediol, 1,4-butanediol, 1,6-hexanediol and glycerol. Bio-based dicarboxylic acids include succinic acid, itaconic acid and adipic acid. All the considered monomers can be industrially produced from renewable feedstock through fermentation or other technologies. [12] It must be also underlined that enzymatically synthesized polyesters are fully biodegradable.

Due to their remarkable catalytic efficiency, enzymes are attractive and sustainable alternative to toxic catalysts used in polycondensation, [13] such as metal catalysts and tin in particular. [14] For instance, lipase B from *Candida antarctica* (CaLB) works efficiently in solvent-free systems and at temperatures below 90°C, [15-17] which are compatible with the polycondensation of unsaturated di-acids that generally suffer from isomerization or cross linking under the harsh reaction conditions requested by conventional chemical processes (i.e. temperatures > 150 °C). [18] Conversely, polyesters bearing reactive functional groups are obtainable and they are prone to further chemical modification or molecular weight enhancement by combining chemical or thermal polymerization [17,18].

Despite the wide array of enzymatic polyester synthesis described in the scientific literature, this wealth of knowledge and catalytic potential is not exploited at industrial scale yet. A recent review of the topic [19] identified biocatalyst efficiency and recyclability among the major problems hampering the implementation of enzymatic polycondensation. Indeed, immobilization of the biocatalyst is mandatory in these synthetic processes: firstly to avoid protein contamination and secondly to allow recycling the expensive enzyme. The latter factor affects severely the economic viability of the process, especially in the case of solvent-free polycondensations where the viscosity of the reaction systems calls for vigorous mixing that can cause mechanical damage of the biocatalyst. [24] Temperatures ranging from 60 °C to 90 °C are also applied to reduce viscosity and improve mass transfer, inferring further stress to the biocatalyst.

The present study analyses the feasibility of solvent-free polycondensation and tries to overcome the major bottlenecks that have caused, so far, the confinement of enzymatic polycondensation at laboratory scale. For the first time, at the best of our knowledge, the problem of contamination of product, caused by enzyme leaching is clearly addressed and commented in relation to reaction rate and polyester elongation. New alternative solutions are here presented that combine the robustness of a covalently immobilized lipase with thin-film processes. Therefore, the study intends to overcome the inadequacy of batch reactions associated with mechanical mixing and to offer a new paradigm for the integrated design of solvent-free enzymatic polycondensation and, more in general, biotransformations involving viscous systems.

Results and Discussion

Covalent oriented immobilization of CaLB.

Most examples of polycondensations reported in the scientific literature make use of a commercial formulation of immobilized lipase B from *Candida antarctica* (Novozym® 435), which has been tested both in solvent-less systems and in the presence of organic solvents. [20, 22, 23, 24] The combination of viscosity and mixing translates into a considerable mechanical stress exerted on the biocatalyst: in a pioneering work, dealing with polycondensation of adipic acid (AA) and 1,4-butanediol (BDO), [1] it was observed that during one single synthetic cycle 10% of the protein detaches from the carrier and contaminates the product. The instability of the anchoring of the enzyme on the support was not solved but just circumvented by adding fresh enzyme after each polycondensation cycle. This drawback is mainly the consequence of the weak anchoring of the lipase on the carrier since the enzyme is immobilized through physical adsorption on a methacrylic resin. [25]

In the present study, we have overcome the problem by using a preparation of CaLB (CaLB-Cov) covalently immobilized on an epoxy-functionalized methacrylic resin. The biocatalyst displays an activity (assayed in the hydrolysis of tributyrin) of 2000 U g_{dry}^{-1}, which is comparable to that expressed by Novozym® 435 (2200 U g_{dry}^{-1}). In order to immobilize covalently the CaLB on the methacrylic carrier and retain the maximum enzymatic activity, the immobilization was performed in the presence of a hydrophobic liquid phase. The immobilization protocol implies the use either of hydrophobic organic solvent (e.g. toluene) or the more environmentally benign rapeseed oil (see Experimental section). As previously discussed [26] and also evidenced through molecular dynamics simulations, [27] aqueous buffers are not the optimal media for immobilizing lipases on organic resins. Hydrophobic interactions between the supports and the active site in principle may hinder the accessibility of the active site of the enzymes. On the contrary, the presence of a highly hydrophobic liquid phase is expected to favor the orientation of the hydrophobic areas surrounding the active site towards the bulk solvent. That conformational behavior induces a higher percentage of proteins to anchor on the support through covalent bonds formed with the residues that are located on the opposite face as compared to the opening of the active site. Ultimately, the immobilization procedure employing hydrophobic media leads to higher immobilization yields. [25]

Preserving the integrity of the biocatalyst by working with a thin film of reaction mixture.

In order to avoid the damage of the carrier described in previous works, [20] no mechanical or magnetic mixing was applied but rather mass transfer was assured by working with a thin film of reaction mixture, as also previously described for pilot scale processes carried out in turbo-reactors. [28] In the attempt of reproducing a thin film on a 10 g lab-scale, a rotary evaporator was used operated at 200 rpm. The application of reduced pressure (70 mbar) facilitated the removal of co-products (e.g. alcohols or water) during the reaction.

The recyclability of the enzyme preparations was evaluated under operational conditions by studying the polycondensation of diethyl adipate (DEA) with BDO (Figure 1) in the presence of 1% of biocatalyst (w/w, referred to the global amount of monomers) and by evaluating the conversion achieved at defined times during each synthetic cycle. Equal enzymatic units of the two preparations (calculated by means of a standard tributyrin hydrolysis assay) were used in the synthesis. The course of the reactions was monitored by exploiting the ^{1}H-NMR signal at δ=1.26 of the methyl group of DEA ($C\underline{H}_3\text{-}CH_2\text{-}O$) and the signal at δ=2.33 ($\text{-}CH_2\text{-}C\underline{H}_2\text{-}C(O)O\text{-}$, the latter assumed constant throughout the reaction. Conversions were evaluated at 10, 20, 40 and 300 minutes over eight recycles.

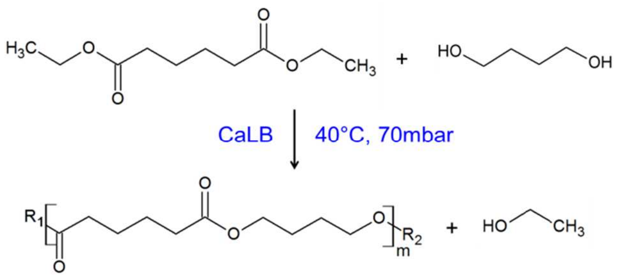

Figure 1. Enzymatic polymerization of diethyl adipate (DEA) and 1,4-butanediol (BDO) at 40 ºC for 5 h under reduced pressure performed using adsorbed and covalently immobilized CaLB preparations. The reaction was employed to study the recyclability of the two enzymatic formulations.

More specifically, Figure 2 shows the conversion of DEA after 10 minutes across eight recycles and more data are available in Figure S1 of Electronic Supplementary Information (ESI).

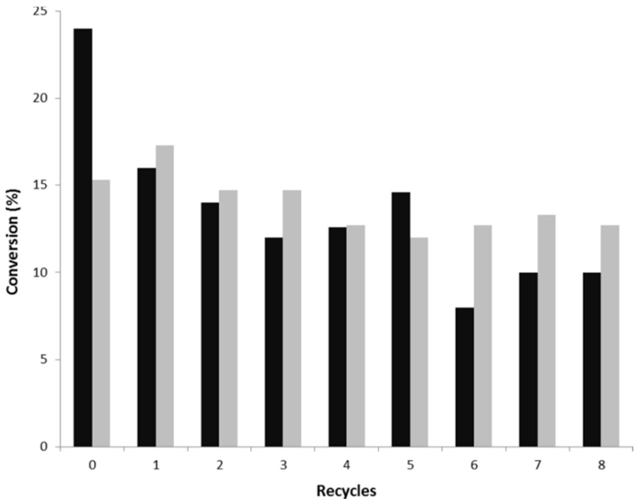

Figure 2. Evaluation of recyclability of the two CaLB preparations over 8 cycles expressed as percentage of DEA monomer reacted after 10 minutes. Novozym® 435: black bars. Covalently immobilized CaLB: gray bars.

The eight recycles were carried out under conditions intended to be closer as possible to industrial needs and sustainability criteria. At the end of each synthetic cycle (300 min) the fluid mixture was filtered without adding any solvent to recover the biocatalysts. Although this procedure implies that some reactant or product, as well as free enzyme, can remain entrapped in the carrier of the biocatalyst, we concluded that the selected procedure provides a more realistic view of the feasibility of the recycling procedure. A plot reporting the weighted moving average of conversions is available in ESI (Figure S1c) and confirms this trend as well as the wider fluctuation of the conversions observed for the reactions catalysed by the adsorbed enzymes. Most probably, that behavior is the consequence of the release of different amounts of native enzyme at various stages of the process, which translates in reaction rates affected by both the immobilized and free lipase. Consequently, the conversions are deeply affected by the uncontrolled leaching and data suggest that the adsorbed CaLB undergoes a first major remarkable decrease in activity already after the first recycle.

Microscope analysis confirmed also the integrity of CaLB-Cov upon recycling (ESI, Figure S2).

Notably, the use of solvents was avoided during the recycling also to avoid potential detrimental effects on the activity of the recovered biocatalysts. A number of solvents were tested and the biocatalysts were rinsed after the synthetic reactions. However, those solvents able to solubilize the reaction mixture caused a loss of hydrolytic activity > 50% (see more details in ESI, Table S1). That can be attributed to a denaturation effect but also to the promotion of enzyme leaching in the case of Novozym® 435.

It was also verified that the amount of biocatalyst used in the process exerts a major effect on the reactivity of DEA. When the same reaction was carried out in the presence of 4% (w/w) of biocatalyst, the observed conversions after 20 min were 71 % for Novozym® 435 and 61% for CaLB-Cov, whereas using 1% of biocatalysts the conversions were 31 and 23 % respectively (see also Figure S1 in ESI for comparison). The reaction catalysed by the adsorbed lipase led to a conversion of 87% after the first hour of reaction and 76% in the case of CaLB-Cov.

Evaluation of enzyme leaching under operational conditions.

Covalently immobilized enzymatic preparations do not automatically assure that enzymes are not released in the product. The protein to be loaded on the support must not exceed the capacity of the functional groups to form covalent bonds. It has been demonstrated that when the immobilized preparations are overloaded, part of the protein is simply adsorbed on the support. [21] Therefore, a balance between the functional groups available on the surface of the polymeric supports and the amount of loaded protein should be achieved for avoiding the release of the enzyme in the reaction mixture. In some cases, the producers of the carrier state the concentration of functional groups on the support (generally in the range of 0.025 to 4.5 mmol per gram of dry polymer). However, it is quite difficult to determine *a priori* the optimum amount of protein to be loaded, since enzymes differ in terms of number of reacting residues and for their molecular size. [29] Washing steps after covalent immobilization are advisable but they do not assure the complete removal of those protein molecules loaded on the carrier *via* simple adsorption, [30] so that the non-covalently bound fraction of enzyme can contaminate the product. Starting from these considerations, the present study addressed the issue of the robustness of the covalently immobilized biocatalyst by evaluating the leakage of active enzyme under different working conditions. Firstly, we determined the activity of the enzyme detached from CaLB-Cov and Novozym® 435 during the course of a standard hydrolytic assay (see Experimental for the complete protocol). Data in Figure 3 clearly show that substantial residual activity can be detected in the liquid phase even after removal of the adsorbed preparation (Novozym® 435) by filtration. No significant residual activity was observed in the case of the CaLB-Cov formulation.

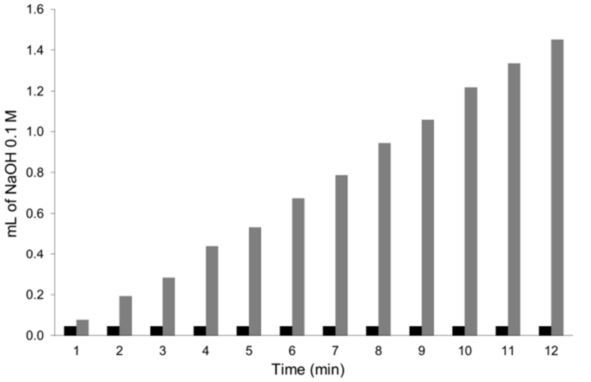

Figure 3. Residual enzymatic activity present in the tributyrin emulsion after incubation of the immobilized enzymatic preparations (15 min at 30 °C) and filtration of the biocatalysts. Legend: black bars= covalently immobilized preparation; gray bars: Novozym® 435. Equal units of the two immobilized enzymes were used in the tests. The hydrolytic activity was determined by titrating the released butyric acid with a 0.1 aqueous solution of NaOH.

Afterward, protein leaching was also evaluated for both enzymatic formulations under polycondensation conditions. Reactions between AA and BDO were carried out at 50 °C for 20 h using the same enzymatic units of the two biocatalysts. The residual active enzyme present in the final product was estimated by titrating the butyric acid released after adding tributyrin directly in defined volumes withdrawn from the reaction mixture at different reaction times (see Experimental Section). Data in Figure 4 show how enzymatic activity is detectable in the reaction mixture throughout the reaction course when the adsorbed preparation (Novozym® 435) was used.

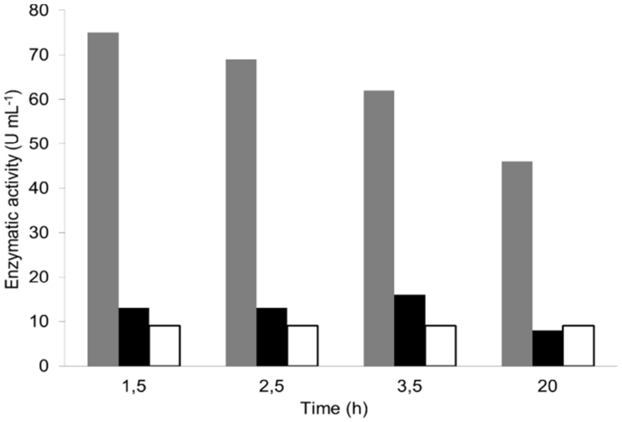

Figure 4. Enzymatic units of free active enzyme present in volumes withdrawn at different reaction times during the polycondensation of AA and BDO and after removal of the immobilized biocatalyst. Legend: gray bars= adsorbed immobilized CaLB (Novozym® 435); black= covalently immobilized CaLB (CaLB-Cov); empty bars: blank.

The maximum of enzymatic activity is observable in the product recovered within the first 3 hours of reactions and this suggests that the largest percentage of enzyme is released at an early stage and then this free enzyme undergo progressive inactivation. On the other hand, the activity of lipase released from the covalently immobilized CaLB is almost comparable to the blank experiments

(Figure 4). The data indicate that the use of adsorbed lipases in polymerization makes unfeasible any analysis of the effect of the biocatalyst on the course of the reaction because active CaLB is present in the reaction mixture both in the immobilized and free form.[31]

It is reasonable to expect that enzyme leaching has a pronounced effect over reaction rates and especially on polymer elongation. Under solvent-less conditions, the viscosity prevents the diffusion of the substrates, and oligomers in particular, into the pores of the carriers. Free enzyme molecules dispersed in the reaction mixture are by far more accessible as compared to the protein anchored or adsorbed onto porous resins.

Effect of enzyme leaching on polycondensation of dimethyl itaconate (DMI) and 1,4-butanediol (BDO) under thin-film conditions.

Itaconic acid (IA) is a renewable monomer that can be produced by fermentation of *Aspergillus terreus*[32] and it represents an interesting monomer due to the chemical versatility of its C=C functional group. Moreover, there is an increasing interest towards IA as monomer for the synthesis of bio-based polyesters because it is the main candidate for replacing maleic and fumaric acids, two largely used petrol-based chemicals currently employed in the production of reticulated polymers.[33] The main drawback of the traditional chemical polymerization of IA resides in the reactivity of the vinyl group at high temperatures (> 150 °C) that causes the isomerization of IA in citraconic and mesaconic acid. Moreover, radical species form with the consequent cross-linking of monomers. Therefore, the use of highly active enzymes at mild temperature represents a route for overcoming these limitations. However, it has been shown that CaLB catalysed polycondensation of itaconic acid derivatives suffer from slow reaction kinetics[18] caused by the poor reactivity of acyl group, which undergoes the stabilizing resonance effect of the conjugated C=C bond.

In order to verify the applicability of the covalently immobilized enzyme to the polycondensation of different monomers, a derivative of itaconic acid, namely dimethyl itaconate (DMI), was considered. The polycondensation was carried out in a thin film in a round bottomed-flask connected to a rotary evaporator operated at 80 rpm at 70 mbar, as described before. At the starting of the reaction, DMI was suspended in the liquid diol (1.0:1.1, molar ratio). The suspension was warmed at 50°C to achieve a fluid slurry. As the reaction proceeded, the mixture became a homogeneous transparent solution. The final product was a viscous sticky colourless liquid that was analysed by means of ESI-MS, and ^{1}H-NMR without any further purification.

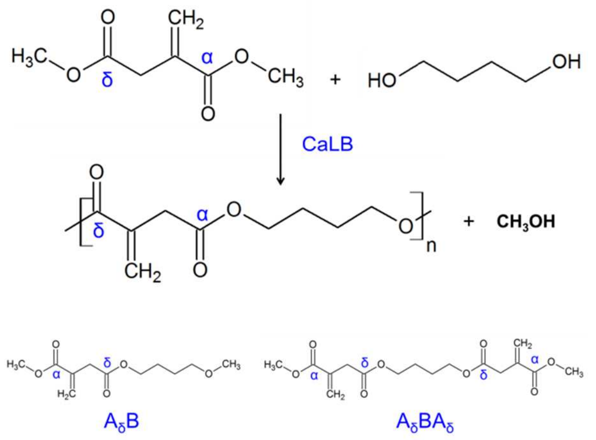

Figure 5. Scheme of the polycondensation reaction between DMI and BDO (top) and structures of the products formed in higher percentage (bottom).

As shown in Figure 6, polyesterification of BDO and DMI catalysed by CaLB-Cov under solvent-free conditions for 72 hours proceeded very slowly and gave a mixture of oligomers between 2 and 5 units with a molecular weight in the range of 216 and 526 m z^{-1}.

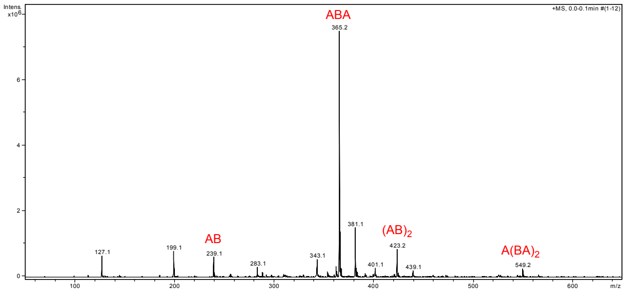

Figure 6. Labelled ESI-MS positive ion mass spectrum of polycondensation products of DMI (A) with BDO (B) catalysed by CaLB-Cov (72 hours).

The major products is represented by ABA trimer where only the fast reacting ester groups of 2 DMI molecules acylate the diol, as also confirmed by ^{1}H-NMR spectrum of products (see ESI, Figure S3). The spectrum indicates that more than 90% of the fast-reactive ester was converted whereas only 2 % of the slow reacting acyl group adjacent to the C=C bond reacted. This can be confirmed by comparing the ^{1}H-NMR signals of the two methoxy groups of DMI. Trimer ABA accumulates because it presents two slow-reacting acyl-groups and, conversely, $(AB)_2$ and $A(BA)_2$ are minor products.

It must be underlined that previous studies[18] described the polycondensation of DMI and different polyols with the formation of products having a M_n ranging from 2000 to 11.900 g mol^{-1}. In that case, Novozym® 435 was employed at 90 °C for 48 hours with the application of reduced pressure only during the last 46 hours of the reaction.

The observed huge difference in the reaction efficiency must be ascribed either to the temperature or to the different formulation of the biocatalyst. All attempts of carrying out the reaction at temperature equal or above

80°C led to the formation of solid products insoluble in all solvents tested (dichloromethane, tetrahydrofurane, acetone, hexane, ethyl acetate, and toluene). This observation suggested that vinyl groups of DMI underwent cross-linking during the reaction course. The polycondensation was attempted both at atmospheric pressure and under reduced pressure and using BDO as diol. Actually, when we reproduced the synthesis of poly(1,4-butylene itaconate) (PBI) at 50°C but using Novozym® 435 (same amount of enzymatic units as in the experiment performed with CaLB-Cov) an array of oligomers having a length up to 18 units were formed (Figure 7). Evidence of the formation of oligomers comes also from the ^{1}H-NMR spectra and the HPLC-DAD chromatogram (see ESI Figures S4 and S5).

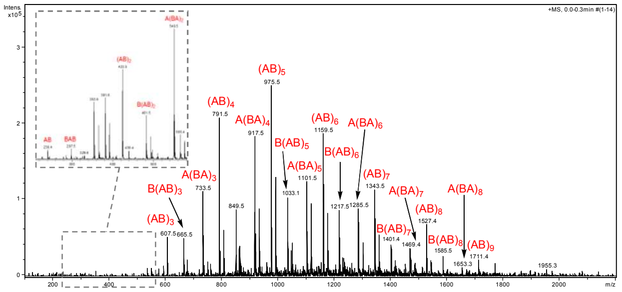

Figure 7. ESI-MS positive ion mass spectrum (100-2000 m z^{-1}) of PBI obtained from the polycondensation of DMI (A) and BDO (B) catalysed by Novozym® 435 (96 hours).

Since the two enzymatic preparations (Novozym® 435 and CaLB-Cov) are endowed with comparable activity (referred to tributyrin hydrolysis), differences in the course of the reactions must stem on the accessibility of the enzymes and, more specifically, on the method of immobilization. It must also be underlined that the chemical nature of the methacrylic resins used as carriers for the two immobilized biocatalysts is quite similar. Data reported above in Figure 4 suggest that the considerable amount of free native CaLB detaches from Novozym® 435 determines a more homogeneous distribution of enzyme molecules in the reaction mixture and, ultimately, favorable kinetics. On the contrary, the amount of lipase leached off the covalently immobilized CaLB is negligible and therefore elongation proceeds with difficulty.

Evaluating the accessibility of covalently immobilized CaLB.

In order to shed light on the lower efficiency of polycondensation catalysed by CaLB-Cov we also explored the chance that the lower accessibility might be ascribed to steric occlusion of the active site of the enzyme due to the covalent bonds between the protein and the carrier. This second hypothesis was evaluated by using the oligomers (PBI) synthesized using Novozym® 435 as a substrate for an elongation reaction where CaLB-Cov was employed as biocatalyst. The elongation was performed by employing dimethyl adipate (DMA), a diester carrying two acyl groups with the same reactivity. DMA and CaLB-Cov (10% wt) were added to PBI and the reaction was carried out under solvent-free conditions, in thin-film at 50 °C and 70 mbar for 72 hours. It must be underlined that no residual enzymatic activity was detected in PBI used for the elongation reaction. The final product was a transparent viscous liquid, and ESI-MS spectra (Figure 8) illustrate how the elongation reaction occurred. ^{1}H-NMR spectra demonstrate the complete acylation of the free hydroxyl groups in the starting oligomers, as indicated by the absence of the signal at 3.5 ppm corresponding to $-CH_2-\underline{CH_2}-OH$. (see ESI, Figure S6) Further evidence of chain elongation comes from HPLC-DAD chromatogram reported in ESI (Figure S7).

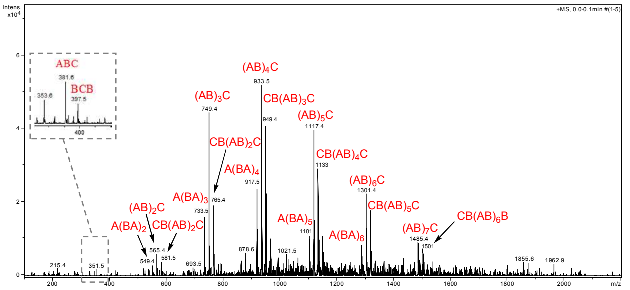

Figure 8. ESI-MS positive ion mass spectrum (100-2000 m z^{-1}) of reaction between PBI and DMA (C) catalysed by CaLB-Cov. Reaction time: 72 hours.

Therefore, the experimental data indicate that even long oligomers can access the catalytic site of the covalently immobilized enzymes. As expected, DMA, which has two acyl functionalities with similar reactivity, leads to faster reaction and products with higher molecular weights.

A further confirmation of the accessibility of the active site of the covalently immobilized CaLB was obtained by studying the hydrolysis of enzymatically synthesized PBI. The hydrolytic reaction was carried out in the presence of 10% CaLB-Cov and 50 °C and compared to a blank experiment without enzyme. HPLC-DAD chromatograms (see ESI, Figure S12) clearly indicate that the PBI olygomers with higher molecular weight are hydrolyzed and the resulting small oligomers accumulate during the course of the reaction.

In conclusion, while the covalent immobilization of the enzyme is necessary for assuring recycling and avoiding contamination, at the same time the low reactivity of DMI requires a homogeneously dispersed enzyme to promote adequate reaction kinetics.

Retrospective analysis of the feasibility of thermodynamically driven polyesterification.

As demonstrated above, enzyme leaching not only determines product contamination but also makes any quantitative analysis of the effect of adsorbed immobilized biocatalysts on the course of the polymerization process unfeasible, because the fraction of free active enzyme present in the mixture (which is also the most accessible) cannot be accounted separately. That observation induce us to shed new light on some data previously reported in patent WO 94/12652,[2] which describes the polycondensation of AA and BDO catalysed by Novozym® 435 in a two-step process. The inventors reported a first oligomerization step followed by the removal of the biocatalyst. Afterward, the reaction continued under heating and reduced pressure with an increase of the M_n (polymerization at 60 °C for 24 h, 10 ± 3 mbar). It is noteworthy that the option of removing the biocatalyst after the synthesis of olygomers would be very attracting since, as the reaction proceeds, the viscosity increases and the recovery of the biocatalyst becomes difficult. However, a later study of the same polycondensation[1] catalysed by a covalently immobilized preparation of CaLB (Chirazyme) reported no increase in M_n during the second polymerization step, and this was taken as the proof that in the first case the polycondensation was simply ascribable to the free enzyme detached from the carrier during the first synthetic step. Unfortunately, Chirazyme is not commercially available any longer, so that in order to confirm that polyester elongation occurs exclusively in the presence of the biocatalyst we made use of the CaLB-Cov formulation.

The two-step synthesis of poly(1,4-butylene adipate) (PBA) was carried out as illustrated in Figure 9.

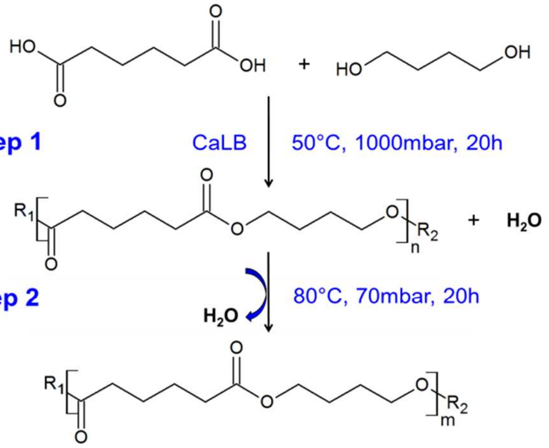

Figure 9. Step 1: Oligomerization between AA and BDO at 50 °C for 20 h in the presence of CaLB-Cov. Step 2: Elongation after removal of the biocatalyst and carried out at 80 °C under reduced pressure.

In the first step, the oligomerization was performed in a syringe for 20 h at environment pressure and 50 °C in the presence of the biocatalyst. No vacuum was applied to this first synthetic step in order to simulate the conditions reported in the previous works.[1, 2] A blood rotator was employed as a mixing system to prevent mechanical damage of the biocatalyst. After filtration of the liquid viscous product and removal of the biocatalyst, the second step was started by increasing the temperature to 80 °C. No mechanical or magnetic mixing was applied but the reaction was carried out in a round-bottomed flask connected to a rotary evaporator operated at 80 rpm under reduced pressure (70 mbar) to facilitate the removal of water formed throughout the polycondensation. Generally speaking, polycondensation of alcohols with carboxylic acids have a not very high equilibrium constant (typically $K_C < 10$, for uncatalysed reaction), so that water must be removed from the reaction mixture in order to obtain a reasonable degree of polymerization.[34] On the other hand, it has been reported that an increase in the alcohol concentration results in a decrease in the reaction rate.[35]

The reaction was monitored by collecting ^{1}H-NMR spectra of the crude product. (see ESI, Figures S9 and S10). The signals of polymerization products were assigned by 2D-^{1}H-TOCSY-^{13}C-HSQC (see ESI, Figure S11). Afterwards, the conversion was monitored by calculating the ratio between ^{1}H signals at δ 3.53 (t, 2H, -CH_2-C<u>H</u>$_2$-OH) and at δ 4.08 (t, 2H, -C<u>H</u>$_2$OC(O).

Table 1. ^{1}H-NMR data used for monitoring the two-step polycondensation of AA and BDO. Step 2 was carried out after removal of biocatalyst.

Catalyst employed	Reaction conditions	Time	Ratio δ 4.08/3.53
Step 1 CaLB-Cov	50 °C	20 h	1.60
Step 2 no	80 °C, 70 mbar	24 h	1.88
Step 2 no	80 °C, 70 mbar	48 h	3.17
Step 2 no	80 °C, 70 mbar	72 h	4.32

Data in Table 1 indicate that during Step 2, despite the absence of biocatalyst, the polycondensation proceeds, although very slowly. The increase in the ratio between signals at δ 4.08 (t, 2H, -C<u>H</u>$_2$OC(O)), which corresponds to the ester formation, and the signal at δ 3.53 (t, 2H, -CH_2-C<u>H</u>$_2$-OH), which corresponds to the free 1,4-butanediol, demonstrates that the esterification occurs. We can presume that this phenomenon was not observed and reported in the previous study[1] because after 20 h the progress of the reaction is negligible, as demonstrated by the ratio of ^{1}H-NMR signals (1.60 *vs* 1.88). Moreover, it must be underlined that the polymerization previously reported was carried out at 60 °C whereas we decided to boost the reaction rate by increasing the temperature to 80 °C.

Models describing the increase of the reaction order as the esterification proceeds have been already reported in the literature, demonstrating that by increasing the M_n the reaction can proceed at lower temperatures.[36] It must be noted that the chemical polyesterification of AA and BDO in solvent-less system generally requires high temperatures (140-160 °C) since the acid needs to be melted to create a homogeneous phase with the diol during the process. Therefore, at the beginning of the enzymatic step 1 (T= 50 °C) the solid AA is only partially solubilized in the liquid BDO whereas, after the first oligomerization step the product appears a viscous uniform solution and under these conditions the reaction is favored.

Indeed, the fact that a polyesterification proceeds even in absence of the biocatalyst is not surprising. Kinetics of self-catalysed polyesterification reactions of AA and diols have been studied extensively[37] and the mechanisms of polyesterification reactions was illustrated already in 1939.[38] The study concluded that self-catalysed polyesterifications follow third-order kinetics with a second-order dependence on the carboxyl group concentration and a first-order dependence on the hydroxyl group concentration. Later studies [39] demonstrated that hydrogen ions dissociate from the diacid molecules but continue to coordinate weakly to the diacid molecules, suggesting that the self-catalysed polyesterification reactions are promoted by the presence of such hydrogen ions. A detailed kinetic and thermodynamic study of the acid catalysed polyesterification is out of the purpose of this research but experimental data indicate that, once a mixture of olygomers is formed, the carboxylic acid present in the mixture can provide the acid catalyst necessary for the polyesterification.

Experimental

Chemicals and reagents.

Commercial rapeseed oil was used for the immobilization of CalB without any pre-treatment or purification. Dimethyl itaconate (99%), 1,4-butanediol (99%), dichloromethane (≥99.9%, GC grade), deuterated chloroform ($CDCl_3$) (99.8 D-atoms, 0.03% v/v of TMS), tributyrin (98%) and ethyl acetate (≥99.5%) were purchased by Sigma-Aldrich. Acetonitrile (≥ 99.5%) was purchased from Riedel-de-Haën. n-Heptane (98.9%) and all the other solvents and chemicals were purchased from AnalR Normapur. All reagents, except for rapeseed oil, were of analytical grade and were used as received without further purification if not otherwise specified.

Enzymatic preparations.

Novozym® 435 is a commercial formulation of lipase B from *Candida antarctica* (CaLB), adsorbed on a macroporous methacrylic resin. The biocatalyst was kindly donated by Novozymes (DK). The activity, assayed in the hydrolysis of tributyrin, resulted to be 2200 U g_{dry}^{-1}. It has been demonstrated that most of the enzyme molecules of Novozym® 435 are localized in a shell of the bead with a thickness of ~100 μm. [24]

The covalent immobilization of CaLB was carried out according to the following protocol: 2 g of methacrylamide polymer in bead form functionalized with epoxide groups, (Relizyme® EC-EP) particle diameter 200-500 μm; average pore diameter 40-60 nm) was washed and dehydrated with acetone (3x4 mL) on a Buchner filter connected to a vacuum pump. 1 g of the washed and dehydrated polymer is put in a 20 mL vial and 12 mL of hydrophobic liquid phase (either toluene or rape-seed oil) was added. A volume of a commercial solution of Lipozyme CaLB L (Novozymes) corresponding to about 15000 U (TBU) was adjusted to pH 8.0 using a 1.0 M NaOH solution. The enzyme solution was then added to the organic phase and the system was stirred continuously (mechanical stirring) for 48 hours at a temperature of 25 °C. Afterwards, the immobilized enzyme was filtered on a Buchner filter and washed with acetone (3x2 mL) and the excess of acetone was removed under reduced pressure. The synthetic activity of the two preparations resulted to be 43,000 U g_{dry}^{-1} (using toluene) and 48,000 U g_{dry}^{-1} (using rapeseed oil) calculated as described below. The hydrolytic activity (hydrolysis of tributyrin) of the formulation immobilized in toluene resulted to be 2000 U per gram of dry preparation. For the CalB immobilized in rapeseed oil the hydrolytic activity was not assayed due to the interference of residual triglycerides adsorbed on the carrier. For this reason, only the CalB immobilized in toluene was employed in the polycondensation reactions. Water content of both preparations was <5% (w w^{-1}). The residual water content in the final immobilized preparations was determined on aluminum plates. A known amount of biocatalyst was dried at 110 °C for 6 h. Water content is defined as the % of weight loss after drying.

Synthetic activity of Lipases.

The synthesis of propyl-laurate was carried out at 55°C with orbital shacking (250 rpm) in a 20 mL vial using equimolar amounts of lauric acid and 1-propanol (1.2 g and 0.36 g respectively). An amount equal to 30-40 mg of immobilized enzyme was added to the substrates and formation of the ester was monitored by HPLC in the first 15% of conversion (RP-HPLC, C-18 column, mobile phase 100% AcN 0.05% TFA, flow 1 mL min^{-1}, UV-VIS detector, 210 nm). 1 enzymatic Unit is expressed as the amount of enzyme able to catalyse the formation of 1 μmol of propyl-laurate per min at 55 °C.

Assay of hydrolytic activity of lipases.

The activity of enzymatic preparations was assayed by following the tributyrin hydrolysis and by titrating, with 0.1 M sodium hydroxide, the butyric acid that is released during the hydrolysis. An emulsion composed by 1.5 mL tributyrin, 5.1 mL gum arabic emulsifier (0.6% w v^{-1}) and 23.4 mL water was prepared in order to obtain a final molarity of tributyrin of 0.17 M. Successively, 2 mL of K-phosphate buffer (0.1 M, pH 7.0) were added to 30 mL of tributyrin emulsion and the mixture was incubated in a thermostated vessel at 30 °C, equipped with a mechanical stirrer. After pH stabilization, 50 mg of biocatalyst were added. The consumption of 0.1 M sodium hydroxide was monitored for 15-20 min. One unit of activity was defined as the amount of immobilized enzyme required to produce 1 μmol of butyric acid per min at 30 °C.

HPLC analysis.

The polymerization products were analysed by HPLC-DAD using a Phenomenex Gemini-NX C18 5 μm (4.6 mm ID x 250 mm L) column and a Phenomenex Menex IB-Sil C8 5 μm (4.6 mm ID x 30 mm L) pre-column connected to a Gilson HPLC system equipped with diode array detector Agilent 1100 Series and autosampler. The elution of the compounds has been done isocratic using a mixture of ultrapure water (0.05 % trifluoroacetic acid) and AcN (0.05 % trifluoroacetic acid) with a flow rate of 1 $mLmin^{-1}$ and the sample injection volume of 10 μL. The eluting components were detected at 210 and 230 nm. Different gradient concentrations of acetonitrile and ultrapure water were used and the details are reported in the Supplementary electronic Information.

^{1}H-NMR spectroscopy.

^{1}H, ^{13}C, 2D-^{1}H-TOCSY-^{13}C-HSQC (Total Correlation Spectroscopy, Heteronuclear Single Quantum Coherence Spectroscopy) NMR spectra were recorded on a Bruker Avance III Ultra Shield Plus 600 MHz spectrometer operating at 600.17 MHz. The used solvent was $CDCl_3$.
^{1}H-NMR spectra related to polycondensation of DMI were recorded on a Varian® Gemini 200 MHz spectrometer operating at 200 MHz. The used solvent was $CDCl_3$.

Electrospray Ionization Mass Spectrometry (ESI-MS).

The crude reaction mixtures were analysed on Esquire 4000 (Bruker) electrospray positive ionization by generating the ions in an acidic environment. Around 10 mg of sample was dissolved in 1 mL methanol containing 0.1% v v^{-1} formic acid. The generated ions were positively charged with m z^{-1} ratio falls in the range of 200-1000. The subsequent process of deconvolution allows the reconstruction of the mass peaks of the chemical species derived from the analysis of the peaks generated.

Recyclability of CaLB-Cov: polycondensation between diethyl adipate and 1,4-butanediol.

The recyclability study was carried out on a scale of 9.6 mL (9.7 g of monomers) according to the following procedure: DEA (6472 mg, 32 mmol, 6.4 mL) and BDO (3244 mg, 36 mmol, 3.2 mL; monomer molar ratio 8:9) were mixed in a 50 mL round-bottomed flask. The two monomers are liquid and completely miscible. The addition of equal amounts of enzymatic units of the two biocatalysts (110 mg of CaLB-Cov and 100 mg of Novozym® 435, corresponding roughly to 1% in weight referred to the global amount of monomers) started the reaction, which run for 5 h at 40^{o}C under reduced pressure (70 mbar) in the flask connected to a rotary evaporator.
The conversion of diethyl adipate was monitored at 10, 20, 40 and 300 minutes by withdrawing volumes (about 50 µL) of the fluid crude reaction mixture that were dissolved in chloroform-d$_1$ and analysed by ^{1}H-NMR. The ratio between the signal at δ 1.26 attributed to methyl group of ethyl adipate ($C\underline{H}_3$-CH_2-O) and the signal at δ 2.33 (-CH_2-$C\underline{H}_2$-C(O)O-) was exploited to estimate the conversion (see Supplementary Electronic Information for full ^{1}H-NMR assignment and recycles details).
At the end of each synthetic cycle (300 min) the conversion of DEA was evaluated in the range of 76-82%. The products and the unreacted monomers were sufficiently fluid to be filtered under reduced pressure without any addition of solvent. The immobilized biocatalyst (beads diameter 200-500 µm) was fully recovered at the end of the reaction by means of a sintered glass filter (porosity 40-100 µm), equipped with cellulose filters. The biocatalyst was not rinsed in order to prevent the detrimental effects that were observed upon solvent treatments (see ESI, Table S1). The recovered biocatalyst was employed for the following synthetic cycle under the conditions above described by adding the same amount of fresh monomers. It was also verified that no reaction occurred in the absence of enzyme.

Evaluation of free enzyme released from the immobilized biocatalysts during a hydrolytic assay

In order to estimate the enzyme leaching 50 mg of biocatalysts were incubated for 15 min at 30°C under stirring in an emulsion composed as described above (Assay of hydrolytic activity of lipases). The enzymatic preparations were then removed from the media by filtration and the residual activity present in the emulsion was titrated by adding tribuyrin as described above.

Assay of the free active enzyme released in the product during the polycondensation.

Reactions between AA and BDO were carried out at 50 °C for 20 h using the same enzymatic units of the two biocatalysts. The active enzyme present in the final product (protein contamination caused by enzyme leaching from the support) was estimated on defined volumes of reaction mixtures withdrawn at 1.5, 2.5, 3.5 and 20 h. The activity was assayed by following the tributyrin hydrolysis and by titrating with 0.1 M sodium hydroxide the released butyric acid. An emulsion composed by 1.5 mL tributyrin, 5.1 ml gum arabic emulsifier (0.6% w/v) and 23.4 mL water was prepared in order to obtain a final molarity of tributyrin of 0.17 M. Successively 2 mL of Kpi buffer (0.01 M, pH 7.0) was added to 30mL of tributyrin emulsion and the mixture was incubated in a thermostated vessel at 30°C, equipped with a mechanical stirrer. After pH stabilization, 100 µL of the reaction mixture (olygomers) was added. The consumption of 0.1 M sodium hydroxide was monitored for at least 30 min to evaluate the residual active enzyme present in poly(1,4-butanediol adipate) oligomer.
In order to exclude the interference of poly(1,4-butylene adipate) during the titration, hydrolysis tests have been performed using a chemically synthesized poly(1,4-butylene adipate) which has been considered as a blank preparation. Blank data showed that the polyester does not interfere with the assay, since no enzymatic activity was detected during these experiments.

Enzymatic synthesis of PBI: polycondensation of dimethyl itaconate and 1,4-butanediol.

Dimethyl itaconate, (35 mmol), BDO (38,5 mmol) and the biocatalyst CalB-cov (10 % w w^{-1} with respect to the total amount of monomers) were mixed in a 250-mL reaction flask and the reaction proceeded connected with a rotary evaporator under reduced pressure (70 mbar) at 50 °C. The molar ratio of diester and polyol used was 1.0:1.1. During the polymerization process the biphasic system becomes a monophasic homogeneous transparent solution. The final product was a viscous sticky colorless liquid, which was solubilized in DCM. After solvent evaporation, the crude product was analysed by HPLC-DAD, ESI-MS and ^{1}H-NMR without any further purification. It was also verified that no reaction occurred in the absence of enzyme.

Enzymatic synthesis of poly(1,4-butylene itaconate-*co*-adipate (PBIA)

An equimolar amount of DMA (referred to IA) was added to PBI synthesized as described here above. The reaction was started by adding 10% (wt) of CaLB-Cov. The reaction was carried out under solvent-free conditions on thin-film at 50 °C and 70 mbar for 72 hours. It must be underlined that no residual enzymatic activity was detected in PBI used for the elongation reaction. The product was a transparent viscous liquid, characterized by HPLC-DAD, ESI-MS and ^{1}H-NMR without any further purification after solubilization in dichloromethane and filtration.

Enzymatic hydrolysis of PBI

90 mg of a mixture of PBI (previously synthesized from DMI and BDO in the presence of Novozym® 435) was dissolved in 1 mL AcN, followed by the addition of 1 mL potassium phosphate buffer 0.1 M pH 7.0. The hydrolysis started at the addition of 10% wt CaLB-Cov (9 mg). The reaction was performed at 50 °C and atmospheric pressure for 5 hours. Control reaction without enzyme was performed in the same conditions. The product was analysed by HPLC-DAD and ESI-MS without any further purification.

Synthesis of poly(1,4-butanediol adipate): Step 1

Adipic acid (9.85 g, 67 mmol) and 1,4-butanediol (6.35g, 70 mmol) (scale 16 g, ratio 1.0:1.1 mol/mol) were mixed in a glass vial and homogenized under magnetic stirring in a solventless system. The product was transferred in a plastic syringe and the addition of immobilized enzyme (1% w/w) started the reaction that run for 20h at 50°C under blood rotator mixing. The final product (oligomer) is a viscous colorless liquid, which can be recovered after filtration of the biocatalyst. No precipitation or purification was performed. All the reactions were performed considering the same units of enzyme calculated on the basis of tributyrin hydrolytic assay.

Synthesis of poly(1,4-butanediol adipate): Step 2

The oligomer produced in Step 1 was recovered after filtration and placed in round-bottomed flask connected to a rotary evaporator under reduced pressure (70 mbar) at 80°C for 20h without biocatalyst. The final product was a white waxy solid at room temperature. About 100 mg of crude product was dissolved in chloroform-d and analysed by ^{1}H and ^{13}C NMR, 2D-^{1}H-TOCSY-^{13}C-HSQC NMR.

Microscopy.

The integrity of the beads after the reaction (thin film under reduced pressure and rotavapor operated at 80 rpm) was evaluated by means of a microscope METTLE FP52 (see Electronic Supplementary Information).

Conclusions

The data here reported disclose some factors that have hampered, so far, the feasibility and economic viability of the synthesis of polyesters catalysed by CaLB. Firstly, the use of CaLB adsorbed on organic resins is inappropriate because a considerable amount of free active enzyme is released in the reaction mixture and this fraction is, actually, the most accessible to the substrates. Therefore, in such cases, information regarding reaction kinetic or efficiency of biocatalysts should be analysed with great caution. [40] On the other hand, efficient mixing systems are essential for overcoming the viscosity of solvent-free reactions, although conventional mechanical stirring methods in batch reactors cause severe damage of immobilized biocatalysts.

The present study proposes a new non-conventional approach for overcoming these bottlenecks. By working with thin films of reaction mixtures and robust covalently immobilized CaLB it is possible to preserve the integrity of the biocatalyst while assuring recyclability, efficient mass transfer and continuous removal of co-products under reduced pressure. The concept has been experimentally validated by synthesizing oligoesters of BDO with AA, DEA, DMA and DMI.

In the case of the slow-reacting DMI, results clearly show that elongation depends mainly on the accessibility and distribution of the enzyme in the reaction mixture. Consequently, future investigations should aim at improving the dispersion of the biocatalyst rather than at employing biocatalysts characterized by high activity condensed in small volumes.

Concerning the polyesterification of free AA, novel attention should be paid to the self-catalysed polycondensation of oligomers while tuning the thermodynamics of the reactions through water removal. On that respect, the thin-film methodology is particularly appropriate because it allows the continuous operation under reduced pressure and facilitates mass and heat transfer. Therefore, in principle, the approach can be applied to most biocatalysed process affected by viscosity. [21, 41]

More specifically, the present methodology could overcome the major problems related also to the production at industrial scale of different specialties chemicals such, for instance, emollient esters for cosmetic formulations. Lipase catalysed solvent-less synthesis involving diglycerol, polyglycerol or other polyols, is hampered by viscosity, enzyme leaching and difficulties in shifting the equilibrium of the reaction to achieve total conversion by removal of water. [21, 41, 42] It has been also reported that fixed-bed red reactors encounter pressure drop along the reactor length and stirred tank reactors are particularly unsuitable because they cause the disintegration of the enzyme carrier by strong shear forces. The synthesis of polyglycerol and lauric acid has been described using new alternative reactors such as bubble column, where the damaging of the carrier was not as pronounced as in a stirred tank reactor. However, surface-active compounds promote leaching of a fraction of enzyme adsorbed on the carrier. [21]

In conclusion, the present study indicate an innovative strategy for enlarging the applicability of biocatalysts in different synthesis, which is not based on the simple adaptation of the biocatalyst to standard reactors [42] but rather intends to design jointly the process, the biocatalyst and the reactors according to an integrated vision.

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Notes and References

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† Electronic supplementary information (ESI) available: microscopy analyses, ^{1}H, ^{13}C, 2D-^{1}H-TOCSY-^{13}C-HSQC NMR analyses, HPLC-DAD chromatograms, ESI-MS spectra, HPLC-DAD gradient.

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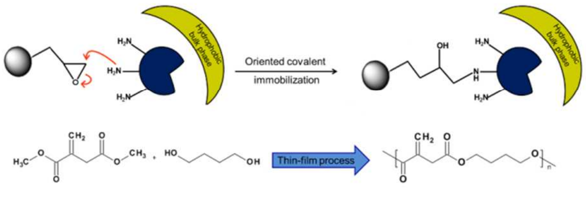

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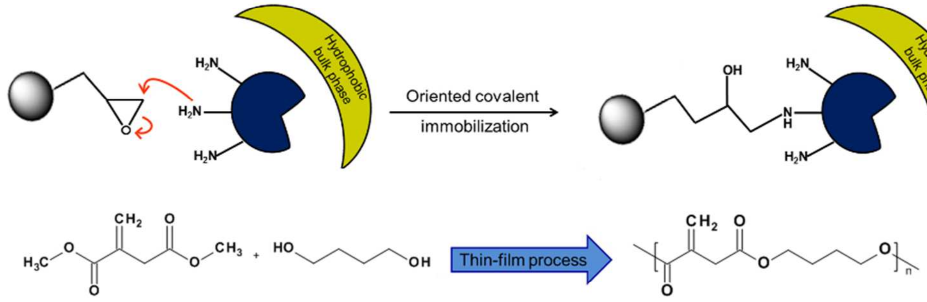


Robust covalently immobilized lipase used in thin-film processes makes enzymes recyclable and imprvoves mass/heat transfer
80x36mm (300 x 300 DPI)

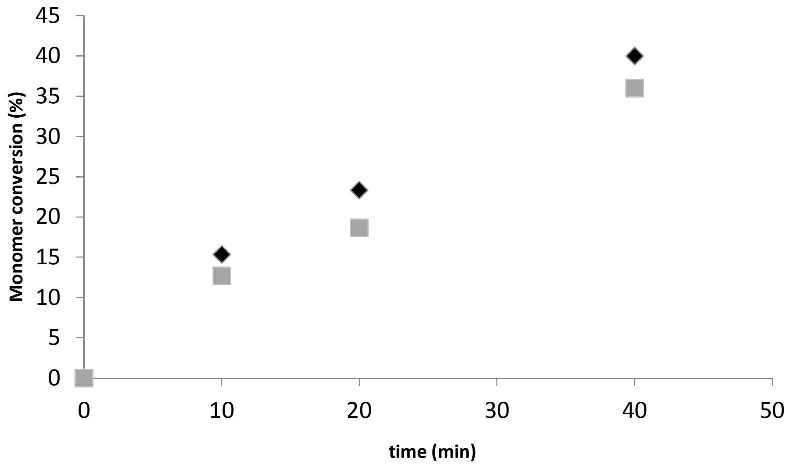
Towards feasible and scalable solvent-free enzymatic polycondensations: integrating robust biocatalysts with thin film reactions

Electronic Supplementary Information

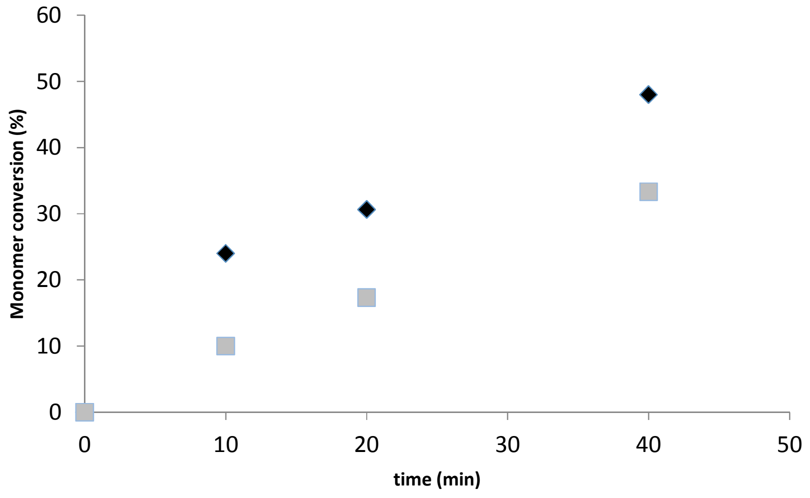
1. Recyclability of the covalently immobilized CaLB

The course of the reaction was monitored by exploiting the ^{1}H-NMR signal at δ=1.26 of the methyl group ($C\underline{H}_3$-CH_2-O) and the signal at δ=2.33 (-CH_2-$C\underline{H}_2$-C(O)O-), the latter assumed constant throughout the reaction. Conversions were evaluated at 10, 20, 40 and 300 minutes over 8 recycles.

^{1}H-NMR (600 MHz, $CDCl_3$): δ 1.26, t, 3H, $C\underline{H}_3$-CH_2-O-; 1.48 t, 2H, -$C\underline{H}_2$-CH_2-OH; 1.56 t, 2H, -$C\underline{H}_2$-CH_2-OC(O); 1.68 t, 2H, -$C\underline{H}_2$-CH_2-C(O)O-; 2.33 t, 2H, -CH_2-$C\underline{H}_2$-C(O)O-; 3.53 t, 2H, -CH_2-$C\underline{H}_2$-OH; 4.08 t, 2H, -$C\underline{H}_2$OC(O); 4.23 CH_3-$C\underline{H}_2$-O- .



a.



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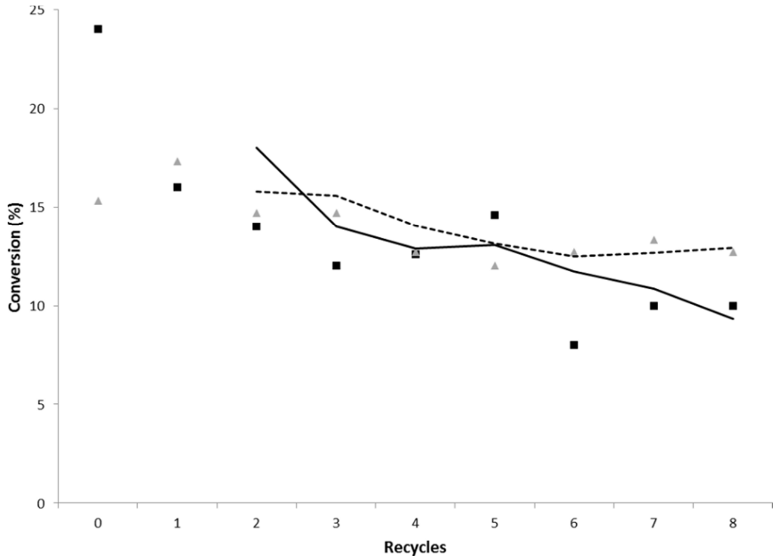

c.

Figure S1. Monomer conversion (extrapolated from ^{1}H-NMR) obtained using CaLB-Cov (a) and Novozym$^{®}$ 435 (b) and evaluated at different reaction times during the first synthetic cycle (black diamonds) and during the 8th cycle (gray squares).

c: Weighted moving average of conversions at 10 min measured after each cycle. Dashed line-triangles: CaLB-Cov. Black line-squares: Novozym® 435. The plot illustrates how Novozym® 435 undergoes a initial severe decrease of activity. Then the biocatalyst undergoes a continuous progressive reduction of activity as well as a wider fluctuation of the observed conversions as compared to CaLB-Cov. That behavior is ascribable to the uncontrolled release of the free enzyme so that the progress of the reaction depends on both the immobilized and free lipase.

Table S1. Solvents tested for their ability to dissolve the reaction mixture and considered as possible candidates for rinsing the biocatalysts during the recycling study. DMSO, 1,4-dioxan and THF resulted able to dissolve both reactants and products but caused a severe reduction of the hydrolytic activity (>50%). The biocatalysts were washed on the filter and under reduced pressure three times with 5 mL of solvent. Then the biocatalyst was rinsed with 10 mL of acetone to remove traces of solvent and dried over the filter before assaying the hydrolytic activity.

Solvent	Monomer mixture	Reaction mixture
THF	soluble	soluble
Acetone	soluble	partially soluble
DMSO	soluble	soluble
1,4-Dioxan	soluble	soluble
DCM	NOT soluble	soluble
Methanol	n.a.	NOT soluble
Diethyl ether	n.a.	NOT soluble

n.a.: data not available

2. Integrity of the immobilized biocatalyst after polycondensation on thin film

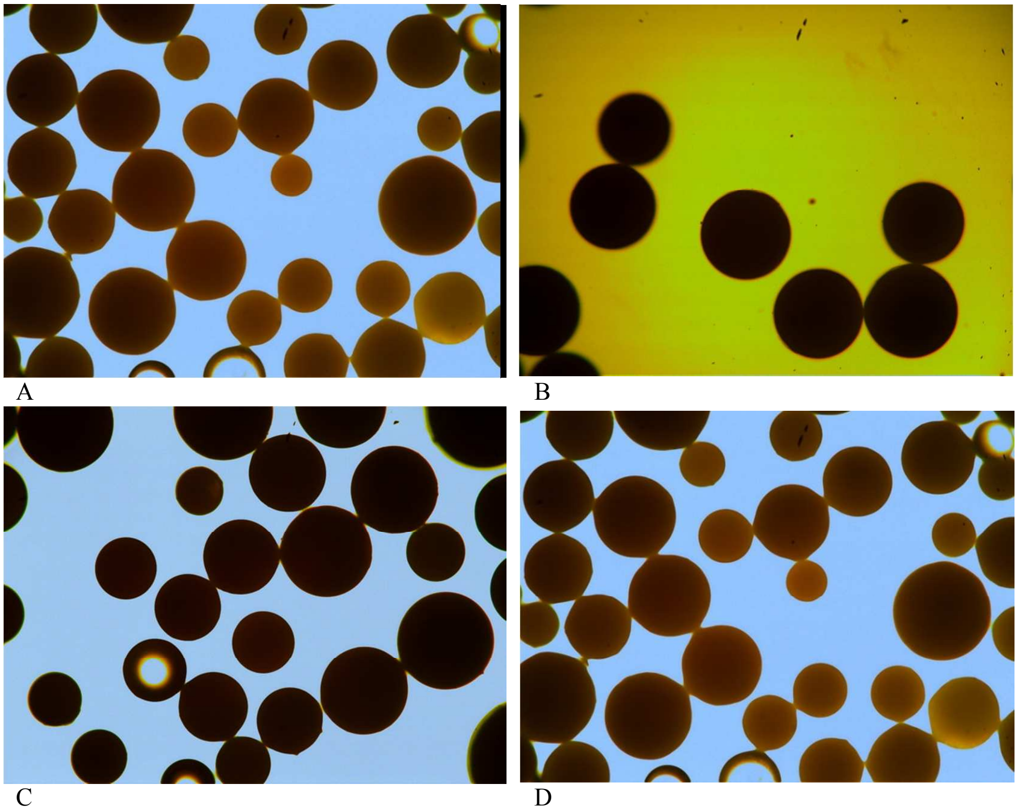

Figure S2. Samples of the covalently immobilized biocatalyst (100 x enlargement, microscope METTLER FP52). Samples A and B: biocatalyst before the synthetic process. Samples C and D: biocatalyst as recovered at the end of 3 cycles of conversion carried out according to the protocol described for the polycondensation of DEA and BDO on 10 g scale, in a round bottom flask connected to a rotary evaporator operated at 200 rpm and at 70 mbar.

3. Polycondensation of DMI and BDO

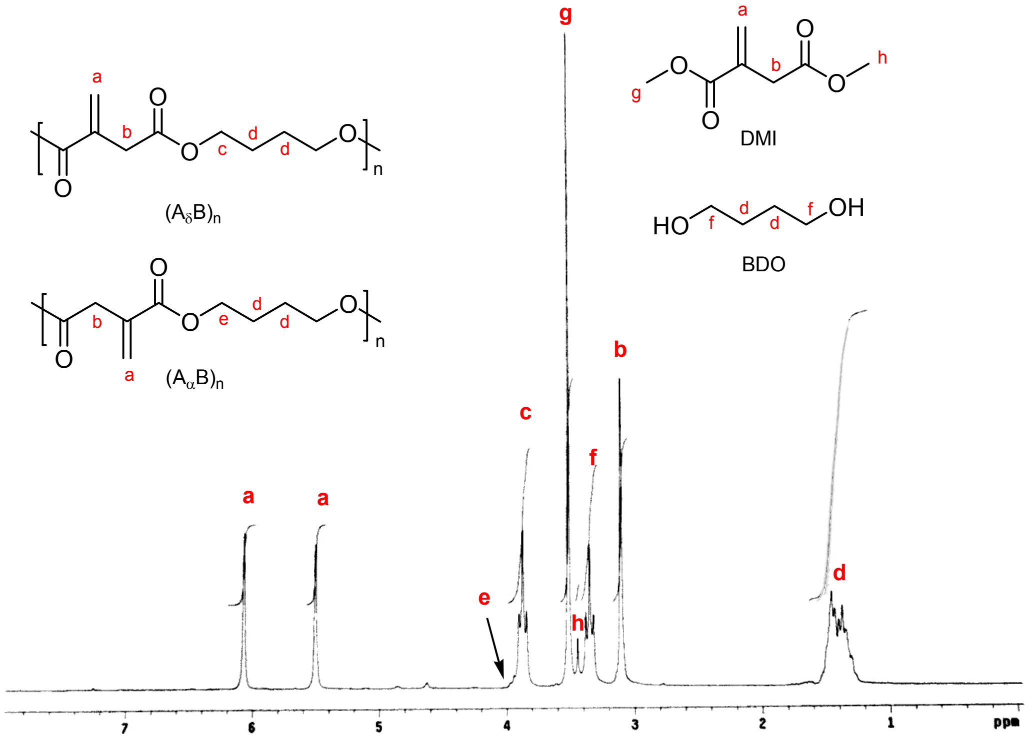

Figure S3. Fully labeled ^{1}H-NMR spectrum of polycondensation product of DMI with BDO catalyzed by CaLB-Cov in $CDCl_3$. Reaction time: 72 hours.

The signals corresponding to the preserved pendant alkene of the itaconate group can be easily distinguished at 5.6 and 6.2 ppm (a). The methylene from the itaconate structure produces a singlet at ~ 3.3 ppm (b). The methoxy groups of unreacted DMI give signals at ~ 3.57 ppm (h) and 3.64 ppm (g), respectively.

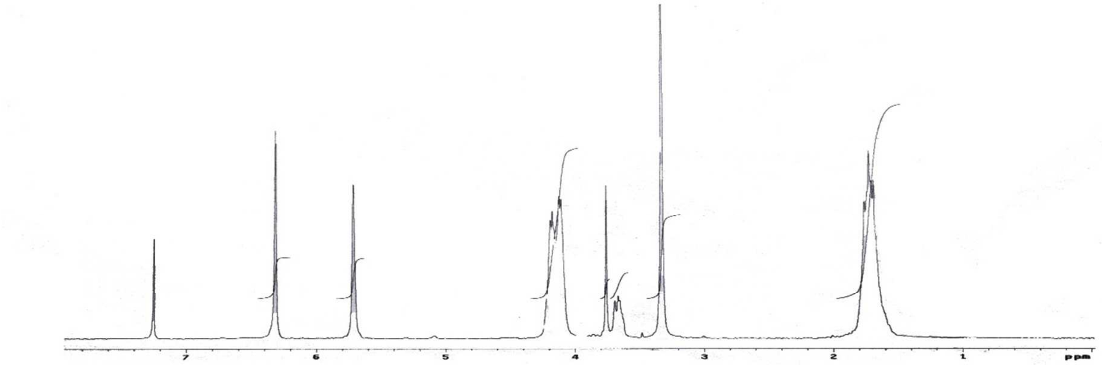

Figure S4. ^{1}H-NMR spectrum of PBI obtained from the polycondensation catalyzed by Novozym® 435. Reaction time: 96 h. ^{1}H-NMR ($CDCl_3$), δ: 1.65 (-C$\underline{H}_2$-CH$_2$-OH), 3.22 ((=C -C$\underline{H}_2$-CO), 3.57 (-CH$_2$-C$\underline{H}_2$-OH), 3.68 (C=C-CO-OC$\underline{H}_3$), 4.19 (-C$\underline{H}_2$-OCO-), 5.65 (-CO- C=C$\underline{H}$H-), 6.27 (-CO-C=CH$\underline{H}$-).

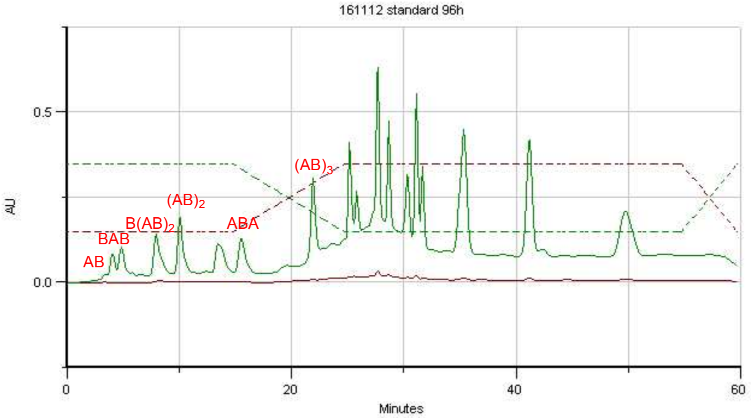

Figure S5. HPLC-DAD chromatogram of polycondensation product (PBI) of DMI with BDO catalyzed by Novozym® 435. Reaction time: 96 hours.

4. Evaluation of accessibility of the active site of covalently immobilized CaLB

4.1 Elongation catalyzed by CaLB-Cov using a PBI oligomeric mixture and DMA

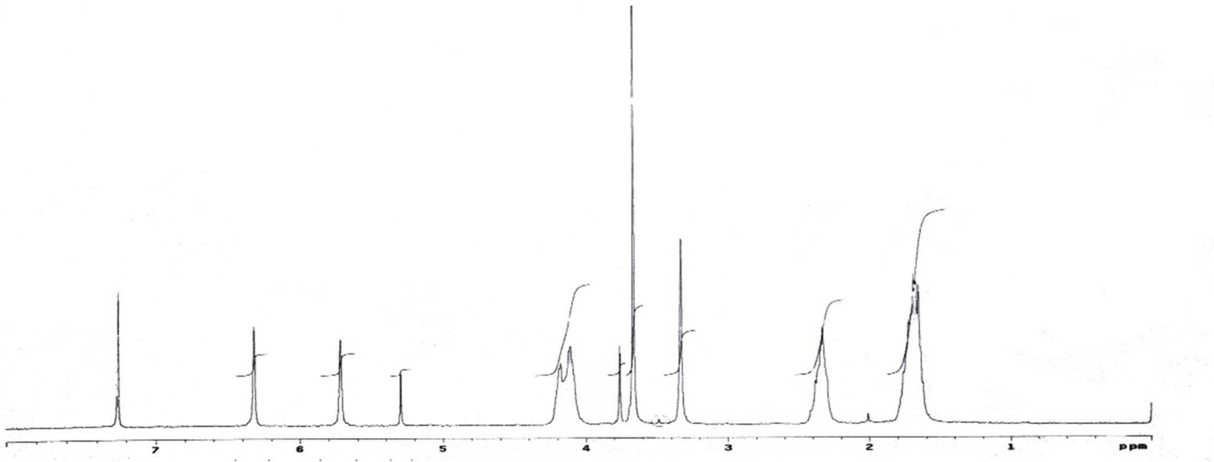

Figure S6. ^{1}H-NMR spectrum of the polycondensation product PBIA catalyzed by CaLB-Cov (elongation step). Reaction time: 72 hours. ^{1}H-NMR ($CDCl_3$), δ: 1.58-1.65 (CH_2-$C\underline{H}_2$-CH_2-CO and -$C\underline{H}_2$-CH_2-OH), 2.24 (2H, t, -CH_2-$C\underline{H}_2$-CO-), 3.22 (=$CC\underline{H}_2$-COO), 3.66 (CH_2-$COOC\underline{H}_3$), 3.69 (C=C-$COOC\underline{H}_3$), 4.19 (-$C\underline{H}_2$-OCO-), 5.65 (-CO-C=$C\underline{H}$H-), 6.27 (-CO-C=$CH\underline{H}$-).

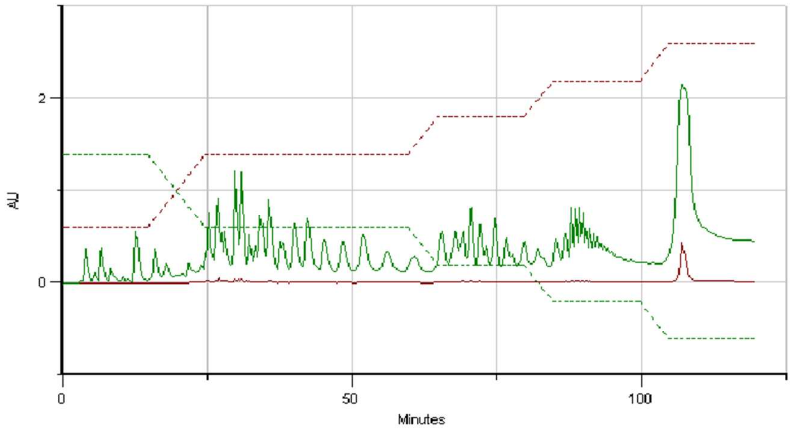

Figure S7. HPLC-DAD chromatogram of polycondensation products of PBI and DMA catalyzed by CaLB-Cov. Reaction time: 72 hours.

4.2 Hydrolysis of PBI catalyzed by CaLB-Cov

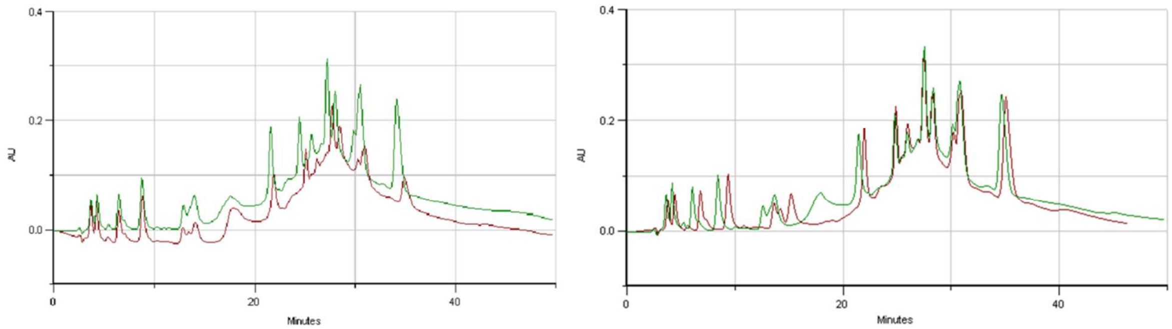

Figure S8. Left: HPLC-DAD chromatogram of hydrolysis product of PBI catalyzed by CaLB-Cov. Green: Time= 0; Red: reaction time 5 hours. Right: HPLC-DAD chromatogram of control reaction without enzyme. Green: reaction time 0 hours; Red: reaction time 5 hours.

5. HPLC-DAD analysis of the polycondensation products

Table S2. HPLC gradient used for the analysis of PBI.

Time (min)	H_2O (% vol)	AcN (% vol)
0	40	60
15	40	60
25	60	40
55	60	40
60	40	60
65	40	60

Table S3. HPLC gradient used for the analysis of PBIA.

Time (min)	H_2O (% vol)	AcN (% vol)
0	40	60
15	40	60
25	60	40
60	60	40
65	70	30
80	70	30
85	80	20
100	80	20
105	90	10
120	90	10

6. Monitoring the two-step polyesterification of AA and BDO via NMR

The progress of the reaction during each step was monitored by calculating the ratio between ^{1}H signals at δ 3.53 (t, 2H, $-CH_2-C\underline{H}_2-OH$) and at δ 4.08 (t, 2H, $-C\underline{H}_2OC(O)$.

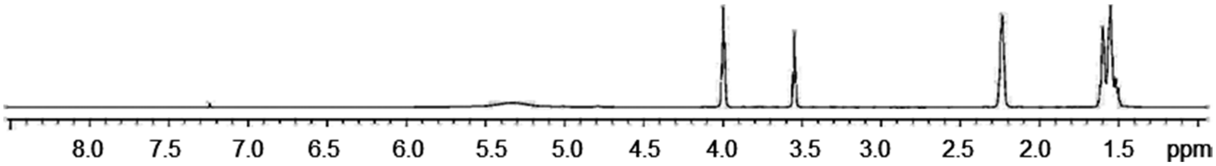

Figure S9. ^{1}H-NMR of products obtained after first step of polycondensation of AA and BDO at atmospheric pressure. ^{1}H-NMR (600 MHz, $CDCl_3$): δ 1.48 t, 2H, $-C\underline{H}_2-CH_2-OH$; δ 1.56 t, 2H, $-C\underline{H}_2-CH_2-OC(O)$; δ 1.68 t, 2H, $-C\underline{H}_2-CH_2-C(O)O-$; δ 2.25 t, 2H, $-CH_2-C\underline{H}_2-C(O)O-$; δ 3.53 t, 2H, $-CH_2-C\underline{H}_2-OH$; δ 4.08 t, 2H, $-C\underline{H}_2OC(O)$.

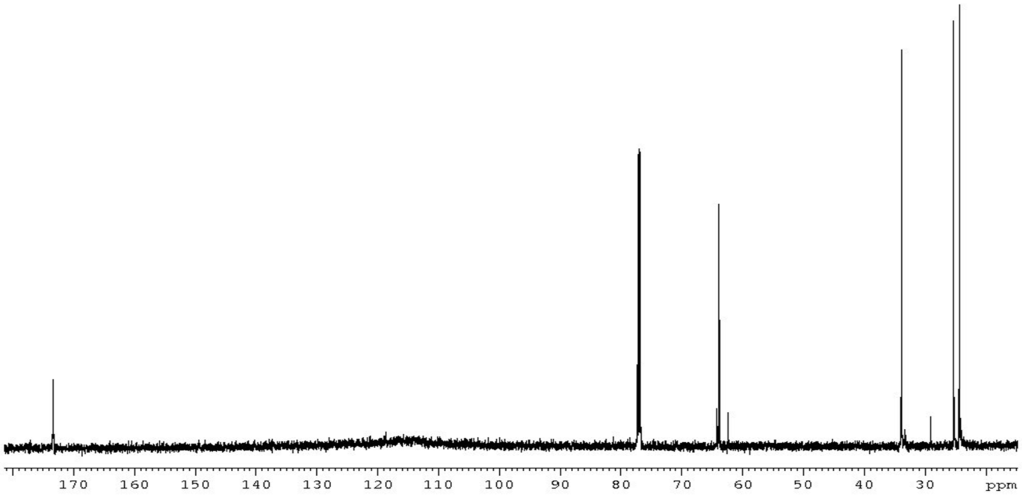

Figure S10. ^{13}C NMR of products of polycondensation of AA and BDO (600 MHz, $CDCl_3$):) δ: 24.3, $-\underline{C}H_2-CH_2-C(O)O-$; 25.1 $-\underline{C}H_2-CH_2-CH_2-OH$; 25.3 $-\underline{C}H_2-CH_2-OC(O)$; δ 29.1 $-CH_2-\underline{C}H_2-CH_2-OH$; 33.9 $-\underline{C}H_2C(O)O$; 62.6 $-CH_2-\underline{C}H_2-OH$; 63.9 $-CH_2-\underline{C}H_2-OC(O)$; 64.1 $\underline{C}H_2-CH_2-CH_2-CH_2-OH$; 173 $-CH_2O\underline{C}(O)$.

The correct assignment of signals of polymerization products was performed by 2D-^{1}H-TOCSY-^{13}C-HSQC.

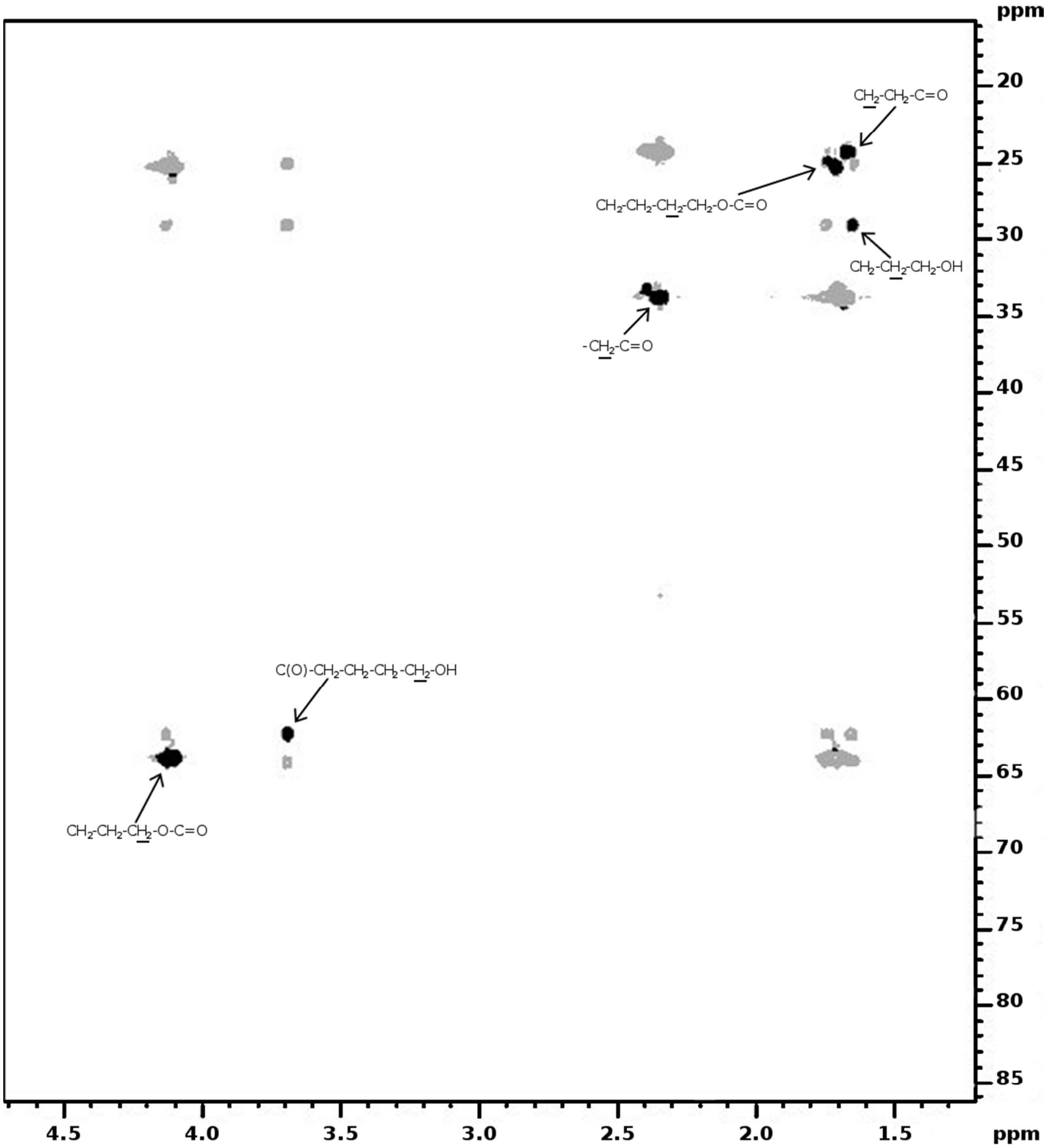

Figure S11. 2D-^{1}H-TOCSY-^{13}C-HSQC of the polymer obtained from AA and BDO following the two steps polycondensation procedure.

The spectra evidences that BDO is correlated with AA demonstrating that the reaction is completed. Once the signals were correctly assigned, it was possible to monitor the reaction progress (for both steps) by measuring the ratio between signals at δ 3.53 (t, 2H, $-CH_2-C\underline{H}_2-OH$) and at δ 4.08 (t, 2H, $-C\underline{H}_2OC(O)$).

List of abbreviations

AA	Adipic acid
AcN	Acetonitrile
BDO	1,4-butanediol
CaLB	*Candida antarctica* lipase B
CaLB-Cov	Covalently immobilized *Candida antarctica* lipase B on methacrylamide polymer
$CDCl_3$	Deuterated chloroform
DCM	Dichloromethane
DEA	Diethyl adipate
DMA	Dimethyl adipate
DMI	Dimethyl itaconate
ESI-MS	Electron Spray Ionization-Mass
HPLC-DAD	High Performance Liquid Chromatography-Diode Array Detector
IA	Itaconic acid
M_n	Number average molecular weight
PBI	poly(1,4-butylene itaconate)
NMR	Nuclear Magnetic Resonance
PBIA	poly(1,4-butylene itaconate-*co*-adipate
PDI	poly(1,4-butylene adipate)
TBU	Tributyrin hydrolytic Units
TOCSY-^{13}C-HSQC	Total Correlation Spectroscopy-^{13}C-Heteronuclear Single Quantum Coherence Spectroscopy